

2-AMINOETHYLPHOSPHONIC ACID IN *MONOCHRYISIS*

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(Text-figs 1-2)

2-Aminoethylphosphonic acid has been identified in a combined form in the lipid and protein fractions of the phytoplankton *Monochrysis lutheri* Droop.

Identification of this compound in marine sources has hitherto been confined to the animal kingdom.

INTRODUCTION

The first naturally occurring compound to be identified containing a carbon-phosphorus bond was 2-aminoethylphosphonic acid,



(AEP), isolated by Horiguchi & Kandatsu (1959) from the lipid fraction of rumen protozoa.

Since then this compound has been shown to be present either in the free state or combined in proteins or lipids in the protozoan *Tetrahymena pyriformis*, freshwater molluscs, terrestrial mammals and marine animals in the phyla Coelenterata, Mollusca and Echinodermata. The distribution of the compound has been reviewed by Quin (1966), and notable additional marine sources reported by Hori, Arakawa & Sugati (1967).

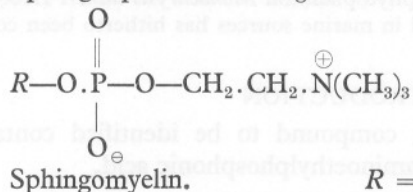
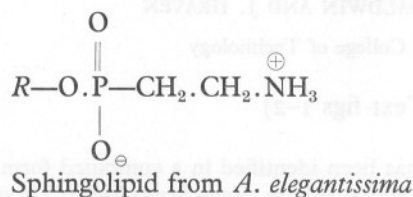
Other naturally occurring compounds containing the carbon-phosphorus bond are α -amino- β -phosphonopropionic acid isolated by Kittredge & Hughes (1964) from *Zoanthus sociatus* and *N*-methyl, *N*-dimethyl, and *N*-trimethylaminoethylphosphonic acid isolated by Kittredge, Isbell & Hughes (1967) from *Anthopleura xanthogrammica*.

2-Aminoethylphosphonic acid incorporated in lipid has been investigated in two cases. Rouser, Kritchevsky, Heller & Lieber (1963) isolated a sphingolipid from the sea anemone *Anthopleura elegantissima* and showed that it was a sphingomyelin with the choline residue replaced by ethylamine.

Subsequent work (Simon & Rouser, 1967) confirmed the structure as 1-(*O*-phosphonoethylamine)-*N*-acylsphingosine. Moreover, it was shown that sphingomyelin itself was absent from the anemone. Quin (1965) has shown that AEP is incorporated in the insoluble protein of *Metridium dianthus* and is not present merely as a result of inadequate extraction of the lipid material.

The biochemical role of AEP and the mechanism of its synthesis by living organisms is still unknown. Rosenberg (1964) has established that inorganic

phosphate provides the phosphorus in the AEP present in the protozoan *Tetrahymena pyriformis*. Harkness (1966) has shown that certain bacteria strains are able to utilize the phosphorus of AEP, as well as certain other aminoalkyl-phosphonic acids, for growth. The ability to catabolize the carbon—phosphorus bond seems widespread among bacteria.



It has also been shown that AEP placed in the diet of the rat (Kandatsu, Horiguchi & Tamari, 1965) or injected intravenously into the goat (Kandatsu & Horiguchi, 1965) was incorporated in the liver lipids of these animals.

Little attention seems to have been paid to the plant kingdom as a source of AEP, even though both unicellular and multicellular algae are important sources of food for marine animals. In view of the previously mentioned incorporation studies in animals, the possibility that the widespread occurrence of AEP among marine animals might be related to its presence in plant foods seemed worthy of examination. As a preliminary we therefore attempted to test the phytoplanktonic organism *Monochrysis lutheri* Droop as a source of AEP, as this plant is well known as a suitable diet for several filter-feeding species of marine animals.

EXPERIMENTAL

The bacteria-free sample of *Monochrysis lutheri* (Millport no. 60) used in this work was kindly supplied by Dr M. R. Droop of the Marine Station, Millport. The cells were harvested during the log-phase of growth.

Extraction and hydrolysis of the lipid fraction

Monochrysis lutheri (4.45 g of dried cells) was homogenized with 200 ml. of chloroform-methanol (2:1, v/v) in a blender for 5 min. The homogenate was centrifuged for 10 min. at 500g, the centrifugate collected and the homogenization repeated with a further 3 × 220 ml. of solvent. The combined centrifugate was evaporated to dryness, the residue extracted with 50 ml. of chloroform, and the solution filtered and evaporated to dryness. Final traces of moisture were removed by vacuum desiccation over solid KOH.

Yield of lipid, 0.753 g.

The total lipid extract was hydrolysed with 25 ml. of boiling 6 N-HCl for 8 h. Water (125 ml.) was then added to the hydrolysate, which was then extracted with chloroform (60 ml.). The aqueous layer was filtered, evaporated to dryness and redissolved in 2 ml. of 0.5 N-acetic acid.

The hydrolysate was then applied to a column of Amberlite ion-exchange resin (IRA 400) in the acetate form equilibrated with 0.5 N-acetic acid and eluted with the same solvent. Fractions (2 ml.) of the eluate were collected using an automatic fraction collector. Tubes 4-9 inclusive contained ninhydrin positive material. The contents of these tubes were then united, evaporated to a volume of 1 ml. and the solution then chromatographed on Whatman no. 1 paper using a descending development.

Two solvent systems were used: phenol/water (4:1) and *n*-butanol/acetic acid/water, (4:1:2) (Horiguchi & Kandatsu, 1960).

In order to achieve the maximum separation of the hydrolysate components, the solvent front was allowed to run to a distance of 38 cm. from the origin. Ninhydrin in butanol was used as a locating agent.

Examination of the residue after lipid extraction

The residue resulting from the chloroform-methanol extraction was collected and dried and 2.3 g hydrolysed with 90 ml. 6 N-HCl under reflux for 8 h. Water (300 ml.) was then added to the hydrolysate and the whole evaporated to a gummy consistency. This residue was dissolved in 300 ml. of water and extracted with chloroform (60 ml.). The aqueous layer was evaporated to a volume of 10 ml., decolorized with Norit, and the volume reduced to 2 ml. This solution was then applied to a column of Amberlite resin IR 120 (H) and the column eluted with water until the eluate was free from chloride ions. The column was then eluted with 300 ml. of ammonia solution (1:9, v/v) and the eluate evaporated to dryness, giving a pale brown solid.

300 mg of the hydrolysate were dissolved in 12 ml. of water containing 0.704 g. of sodium bicarbonate and a solution of 1.4 g of 1-fluoro-2,4-dinitrobenzene in 12 ml. ethanol was added. The mixture was shaken at room temperature for 8 h and the alcohol then evaporated. Water (15 ml.) was then added and the aqueous solution extracted with water-saturated ether until the ether extract was colourless. The aqueous solution was concentrated to a volume of 2 ml. and the pH adjusted to 1 by the addition of N-HCl. The precipitate was removed by centrifugation and the aqueous phase adjusted to a volume of 20 ml. by the addition of water. This aqueous solution was extracted 4 times with water-saturated ether, and then 4 times with water-saturated ethyl acetate. The ethyl acetate solution was dried over anhydrous sodium sulphate and evaporated to a very small volume. Samples of this solution were chromatographed on thin layers of silica gel (Merck) using three different solvent systems: *n*-propanol/35% aqueous ammonia (7:3); ethanol/35% aqueous ammonia (7:3); and *n*-butanol saturated with 35% aqueous ammonia. Each system indicated that DNP-AEP was present in the mixture.

Marker compounds for chromatography

2-Aminoethylphosphonic acid was prepared from 2-bromoethylphthalimide and triethylphosphite according to Kosolapoff (1947), m.p. 282-284 °C.

N-2,4-Dinitrophenylaminoethylphosphonic acid was prepared according to Quin (1965).

RESULTS

Lipid fraction

Paper chromatography of the acid hydrolysate of the chloroform-soluble lipid extract indicated that there were eleven ninhydrin-positive compounds present (see Fig. 1). One of these compounds had the same R_F value as AEP in the two solvent systems used. Moreover, chromatography of a mixture of the hydrolysate and AEP did not result in any resolution between the authentic AEP and the appropriate compound of the hydrolysate. In view of the small quantity of material available no attempt was made at isolation: the AEP was a minor component of the hydrolysate.

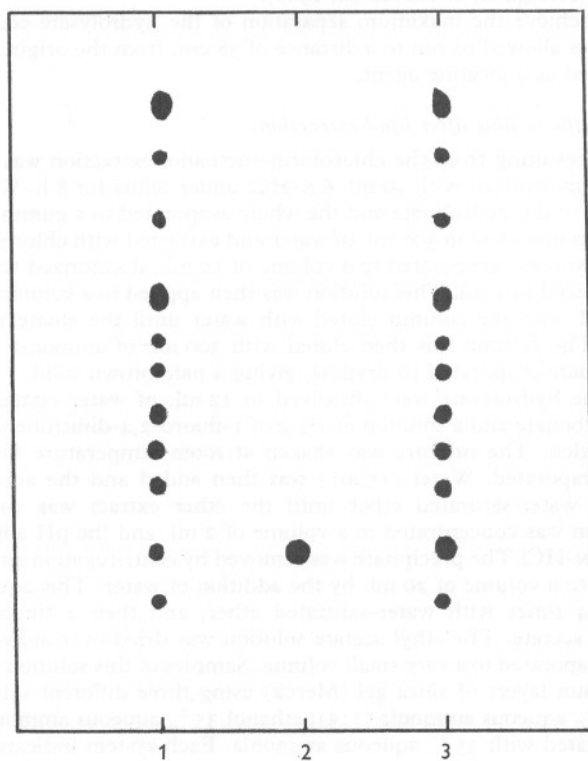


Fig. 1. Paper chromatogram of the hydrolysate of the lipid fraction of *Monochrysis lutheri*. Solvent system: *n*-butanol-acetic acid-water (4:1:2). 1, Hydrolysate; 2, AEP; 3, 1 and 2.

Protein fraction

Amino acid analysis of mixture containing AEP can be readily achieved using automated equipment. In view of the lack of such equipment the

alternative procedure of dinitrophenylation was used to examine the proteinaceous residue. Because of the unusual properties of *N*-2,4-dinitrophenyl-AEP (DNP-AEP) (Quin, 1965), it is possible to separate DNP-AEP from the bulk of the DNP-amino acids, thus facilitating chromatographic analysis. Using this method, AEP was detected in the hydrolysate of the protein fraction by paper chromatography (see Fig. 2).

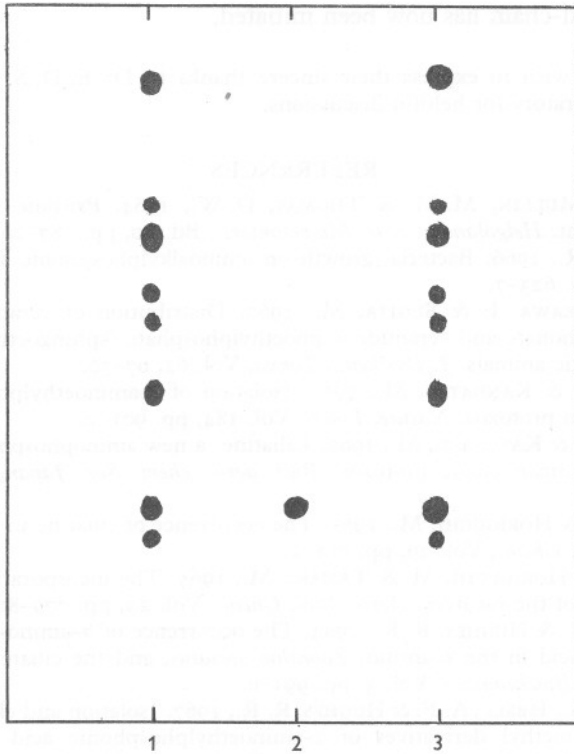


Fig. 2. Thin-layer chromatogram (silica-gel) of the extracted DNP-amino acids from the protein hydrolysate of *Monochrysis lutheri*. Solvent system: *n*-propanol-35% aqueous ammonia (7:3). 1, DNP-hydrolysate; 2, DNP-AEP; 3, 1 and 2.

CONCLUSIONS

As previously stated, AEP can be incorporated into animals via the diet. The detection of AEP in the unicellular alga *Monochrysis lutheri* indicates that the presence of this compound in higher marine animals could possibly have arisen as the result of dietary incorporation.

If this proposition is true, then there arises the possibility that AEP may prove useful as a biochemical integrator for phytoplankton assimilation by marine animals, especially zooplankton.

A compound previously suggested as being suitable for such indicator studies is pristane (Blumer, Mullin & Thomas, 1964). This hydrocarbon, occurring in concentrations of up to 3% of the body lipid in certain zooplankton, is presumed to arise in these animals from dietary phytol and to accumulate at a rate dependent on the quantity of chlorophyll ingested, thereby serving as an indicator of phytoplankton assimilation.

An investigation of a similar nature but using AEP instead of pristane to follow the food-chain has now been initiated.

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